1988

Development of an Analytical Method for the Trace Determination of Mycophenolic Acid by High Performance Liquid Chromatography Following Extraction of the Compound from Feedstuffs

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DEVELOPMENT OF AN ANALYTICAL METHOD FOR THE TRACE DETERMINATION OF MYCOPHENOLIC ACID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOLLOWING EXTRACTION OF THE COMPOUND FROM FEEDSTUFFS

By

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A thesis submitted
in partial fulfillment of the requirements for
the degree of Master of Science
Major in Chemistry
South Dakota State University
1988

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FOR THE TRACE DETERMINATION OF MYCOPHENOLIC
ACID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is accepted for meeting the thesis requirement for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily those of the major department.

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to the faculty and staff of the Chemistry Department and especially to the following people:

Dr. Duane Matthees for his guidance and humor which helped me during the course of the research work.

Dr. David Hilderbrand for his suggestions and leadership.

Nancy Skyberg for typing this thesis.

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STATEMENT OF PROBLEM

Mycophenolic acid is toxic to animals consuming it beyond a certain level and possesses antitumor activity. Not many methods for its trace analysis in feedstuffs and agricultural products have been reported. Thus, it is desirable to develop a standard analytical technique for such important compounds. The purpose of this investigation is to develop a technique or method sensitive enough to determine trace quantities of the compound.

The method developed is based on solvent extraction from feedstuffs and detection by high performance liquid chromatography. Solvent extraction methods have given good results with low background interferences when cleaned up by column chromatography and/or acid base partitioning (for some grain samples only). High performance liquid chromatography was chosen for the detection of mycophenolic acid because it offers a high degree of sensitivity and selectivity with very few background interferences when compared to gas chromatography.

LITERATURE REVIEW

Mycophenolic acid, a metabolite of several species of Penicillium,[1] with a molecular formula $C_{17}H_{20}O_6$, and with the structure

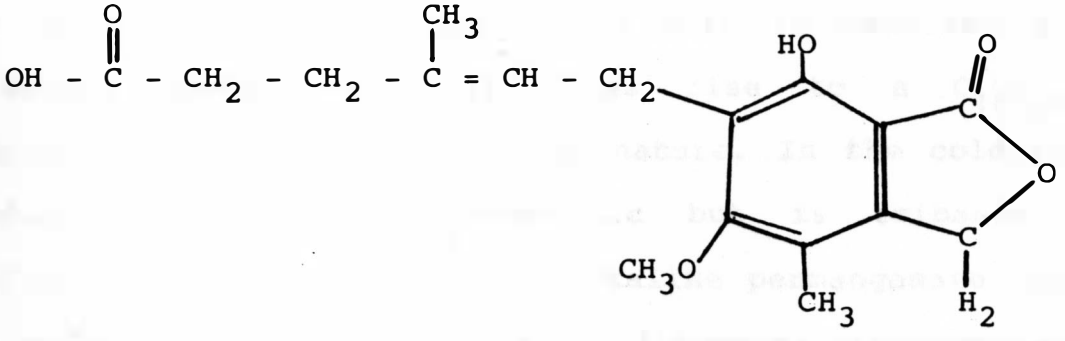

Figure 1. Structure of Mycophenolic Acid

was the first antibiotic substance purified and crystallized by Gosio in 1896.[2]

The structure of mycophenolic acid as described by Wilson is presented in the following discussion.[3] The compound has a -COOH group which can be esterified and which is titratable in the cold, a lactone ring which is titratable in the cold, and the grouping C = C which readily adds two atoms of iodine. Palladium and hydrogen quickly reduce the compound to give dihydromycophenolic acid ($C_{17}H_{22}O_6$). Mycophenolic acid is stable to both hot

acids and alkaline solutions and it titrates as a dibasic acid. It gives a characteristic precipitate with bromine water. Its heavy metal salts are insoluble in water, whereas the potassium salt is soluble in water but insoluble in ethanol.

Fusion of mycophenolic acid with potassium hydroxide gives 1,5-dihydroxy-3,4-dimethyl benzene. When the methyl ether of mycophenolic acid is oxidized with potassium permanganate, it gives rise to a $C_{18}H_{20}O_8$ product which is not ketonic in nature. In the cold this product titrates as a monobasic but is tribasic on heating. Further oxidation in alkaline permanganate gives the anhydride of 1,5-dimethoxy-2,3,4,6-tetracarboxybenzene.

Oxidation of mycophenolic acid with nitric acid gives an oily compound, $C_7H_{10}O_4$, containing both a lactone ring and a carboxyl group. This acid partially isomerizes in alcoholic sodium hydroxide to give a dibasic acid, $C_7H_{10}O_4$. This product is oxidized with difficulty using permanganate to give only oxalic and acetic acid.

Ozonizing mycophenolic acid ($C_{17}H_{20}O_6$), its methyl ether ($C_{18}H_{22}O_6$) and monomethyl methyl ester ($C_{19}H_{24}O_6$) give the following products when decomposed with water. levulinic acid, $CH_3CO(CH_2)_2COOH$, and an aldehyde, $C_{12}H_{12}O_5$, containing one OCH_3 group giving the same ferric chloride reaction as the parent compound are produced. The

monomethyl ether which containes two OCH_3 groups furnishes levulinic acid, a $C_{13}H_{14}O_5$ aldehyde with two of the OCH_3 groups and a $C_{13}H_{14}O_6$ carboxylic acid. The monomethyl ether methyl ester with three OCH_3 groups produces methyl levulinate, $CH_3COCH_2CH_2COOCH_3$, and the same $C_{13}H_{14}O_5$ aldehyde. The yield of these products serves as proof that mycophenolic acid contains a free carboxyl group attached as a side chain, which is the source of levulinic acid from mycophenolic acid, and its monomethyl ether or methyl levulinate from ozonolysis of the monomethyl ether methyl ester.

From this additional evidence mycophenolic acid is described as 6-hydroxy-4-methoxy-3(or 5)-methyl-5(or 3)-(5'-carboxy-3'-methylpent-2'-ethyl) phthalide.

The triclinic structure of mycophenolic acid crystals was reported by Harrison and his co-workers.[3] This structure was determined from diffractometer data by direct methods. It was refined by full-matrix least-squares techniques to R 0.076 for 15478 observed reflections. Two approximately planar sections make up the molecule. These are the ring system and the extended side chain which is at an angle of 79°. The C=C double bond in the side chain has a trans configuration while the bond angles and bond lengths are normal. Molecules of mycophenolic acid are joined by two bonds. One is a single

normal OH---O hydrogen bond between carboxy groups. The other is a single bifuracted hydrogen bond which is partly intra- and partly intermolecular.

The crystals melt at 140°C, are optically inactive, and give an intense blue or blue violet solution upon the addition of ferric chloride. They are nearly insoluble in cold water but may be crystallized from hot water solution. Neither Fehling's solution nor ammoniacal silver nitrate are reduced.[5]

Mycophenolic acid can be synthesised chemically by a convergent synthesis starting from $BrCH_2CH:CMe(CH_2)_2COOMe$ (II) and 5,7-dihydroxy-4-ethylphthalide (III). Condensation of II and III with Ag_2O in dioxane affords the Me ester of nor-O-methyl mycophenolic acid. Selective methylation and hydrolysis gives mycophenolic acid.[6]

Colombo and his co-workers showed the chemical synthesis of mycophenolic acid. The main point in this synthesis is the conversion of 6-farnesyl-5,7-dihydroxy, 4-methylphthalide into mycophenolic acid through the hydroxy ketone of the compound 6-farnesyl-5,7-dihydroxy, 4-methylphthalide by oxidation of the central double bond.[7] A convergent aromatic annulation strategy based on the thermal combination of heterosubstitutes acetylenes and cyclobutenones has been found to be an efficient synthesis of mycophenolic acid.[8]

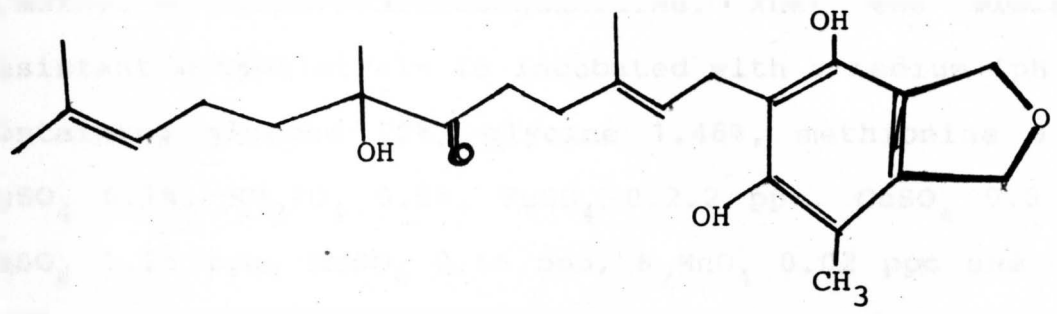

Figure 2. Structure of the Hydroxy Ketone of 6-farnesyl-5,7-dihydroxy,4-methylphthalide.

Most frequently mycophenolic acid is isolated from Penicillium cultures. It is produced by culturing a mutant of Penicillium requiring myconositol. Thus, Penicillium brevi-compactum is cultured with shaking at 27°C for 10 days in a medium containing glucose 10%, glycine 1.46%, methionine 0.50%, KH_2PO_4 0.3%, $MgSO_4$ and K_2MoO_4 and 5 µg myoinositol/ml.[8] It can also be produced by culturing a strain of Penicillium in a medium containing a purine nucleotide. Thus, Penicillium brevi-compactum is cultured at 26°C for 12 days in a medium containing glucose 3.6%, peptone 1.5%, KH_2PO_4 0.06%, $MgSO_4.7H_2O$ 0.04% and guanosine 0.5%.[10]

Mycophenolic acid is also produced by fermentation with viologen-resistant Penicillium strains. Thus, a viologen resistant mutant of Penicillium brevi-compactum is produced by treatment of the parent strain with

N-methyl-N'-nitro-N-nitrosoguanidine. When the viologen-resistant mutant strain is incubated with a medium (pH 6.0) containing glucose 10%, glycine 1.46%, methionine 0.05%, $MgSO_4$ 0.1%, KH_2PO_4 0.3%, $FeSO_4$ 0.2.2 ppm, $CuSO_4$ 0.3 ppm, $ZnSO_4$ 0.25 ppm, $MnSO_4$ 0.16 ppm, K_2MnO_4 0.02 ppm and $CaCO_3$ 1.0% at 27°C for 144 hrs, the accumulation of mycophenolic acid reached 155 mg/dL, compared to 130 mg/dL for the parent strain.[11]

When grown on Czapek-Dox agar Penicillium brevi-compactum produces mycophenolic acid after a vegetative mycelium is formed and as aerial hyphae are developing. Nutrients are not limiting when the synthesis begins. If aerial hyphal development is prevented by placing a dialysis membrane over the growing fungus, no mycophenolic acid is produced. When the dialysis membrane is peeled back and, as a consequence, production of aerial hyphae begins, mycophenolic acid biosynthesis is observed. Apparently, mycophenolic acid is produced only by Penicillium brevi-compactum colonies that possess an aerial mycelium.[12]

Uchida and his co-workers separated the solution containing mycophenolic acid by adjusting the pH to less than 7 to crystallize the compound.[13] With this method 2 l of the fermented solution of Penicillium brevi-compactum containing 1 g of mycophenolic acid is maintained at 50°C,

and the pH is adjusted to 3 with HCl. The solution is cooled gradually to 5°C to precipitate 1.05 g crude crystals containing 0.97 g of mycophenolic acid.

Mycophenolic acid has antiviral activity *in vitro* against vaccinia, measles, herpes simplex, and newcastle disease viruses. Activity is seen at a concentration of 0.5-40 µg/pad and 1 µg/7 mm pad equal to approximately 50 µg/ml. When mycophenolic acid is administered in 9 to 10 doses at 45 to 300 mg/kg each it effectively inhibits the growth of a large number of transplantable murine solid tumors including Walker carcinosarcoma, Mecca lymphosarcoma and Gardner lymphosarcoma. The compound is equally effective when administered at the same dose level either orally or intraperitoneally. Mycophenolic acid also has marginal activity against several leukemia and ascites tumors.[14]

Berman and his co-workers showed the antiviral effect of mycophenolic acid against *Leishmania tropica* in human macrophages. The drug was tested against *Leishmania tropica amastigotes* (mammalian forms) within human macrophages, a model in which achievable serum concentrations of antileishmanial currently in use eliminates approximately 90% of the parasites.[15]

Oral administration of a therapeutic level of mycophenolic acid to rats bearing the Yoshida ascites tumor

resulted in a striking inhibition of the incorporation of hypoxanthine into guanine nucleotides by the tumor cells in vitro.[16]

Mycophenolic acid or mycophenolic monosodium salt completely inhibits the growth of Mecca lymphosarcoma and the growth of the solid and ascites forms of Walker carcinosarcoma 256 and to a lesser extent inhibits the growth of Gardner lymphosarcoma, C3H mammary carcinoma, adenocarcinoma 755, Ridgeway osteogenic sarcoma, X5563 plasma cell myeloma, and the Shionogi androgen-dependent mammary carcinoma 155.[17]

Dyke and his co-workers reported the treatment of hyperuricemia with mycophenolic acid.[18] Oral or parenteral treatment of hyperuricemic patients with daily doses of 200-5000 mg mycophenolic acid or its equivalent of sodium mycophenolate or potassium mycophenolate decreases uric acid levels with relatively no undersirable side effects. In addition, the inhibitory effect of mycophenolic acid on expression of tuberculin hypersensitivity in guinea pigs was reported by Ohsugi and his co-workers.[19]

Mycophenolic acid can occur as a free acid or as mono or disodium salts. Each form has been found to be effective in antiviral and antitumor activities. In particular the disodium salt is found to be effective against the following solid transplanted tumors: sarcoma

180, Lewis lung, carcinoma 755, Walker 256, and Yashida tumor.[20]

All reports indicate that the aromatic portion of the compound is responsible for all the antiviral activities. Most modifications of the compound, such as reduction of the side chain double bond, methylation of the phenolic functionality and slight modification of the aromatic methyl or phthalid methylene group lead to complete inactivation.[21]

The partially harmful aspects of mycophenolic acid result from the fact that it is one of the many metabolites produced by organisms which infect and spoil food materials. This infected food could be toxic to animals consuming it. Daily oral doses of 3600 mg of mycophenolic acid (range 40-80 mg/kg) have been given to psoriatics without any toxicity signal. Toxicity studies of 2 weeks duration indicate that rats and dogs are able to tolerate daily oral doses of 80 mg/kg.[22]

Carter et al., performed an acute toxicity test on mice, rats and monkeys to better assess the untoward effects of mycophenolic acid as a therapeutic agent.[23] Chronic toxicity in the rats and monkeys was tested by daily intragastric administration of freshly prepared mycophenolic acid suspension for 23 weeks and 28 weeks respectively. Rats given 30 mg/kg/day became lethargic and

pale. They lost weight and all were dead within 9 weeks. Heart failure due to anemia was frequently the cause of death. Fifteen mg/kg/day gave only a moderate anemia which improved spontaneously. Male rats were able to sire healthy offspring but the females failed to implant the fertilized ovum. Monkeys given 150 mg/kg/day developed abdominal colic, loss of weight, and bloody diarrhea after 2 weeks' feeding. These signs disappeared upon withdrawal of mycophenolic acid.[23]

The same group also studied the biochemical mechanism for the mitotic inhibition by mycophenolic acid and found that inhibitory levels suppress nucleic acid synthesis in L-cells. Guanine reverses this effect, although hypoxanthine, adenine and xanthine do not. Mycophenolic acid prevents incorporation of labeled hypoxanthine into xanthine and guanine nucleotides but not into adenine nucleotides. The main biochemical action attributable to mycophenolic acid is marked inhibition of inosinic acid dehydrogenase which converts inosinic acid to xanthylic acid.23

The study of Sweeney and his co-workers, indicates that mycophenolic acid is rapidly absorbed from the digestive tract, conjugates with glucuronide to form mycophenolic acid-glucuronide and is excreted as such in the urine.[24]

All the important biological activities of mycophenolic acid make it necessary to determine trace levels of the compound in feed samples and agricultural commodities. This necessity leads to the search for sensitive and selective methods for the separation and determination of mycophenolic acid. Solvent extraction is used for eliminating interferences and also for concentrating the analyte. In solvent extraction procedures selectivity can be increased by full or total control of pH and choice of solvent.

Mycophenolic acid is determined by different analytical methods and techniques. Of the analytical methods employed for the determination of mycophenolic acid the chromatographic method is widely used.

Determination of mycophenolic acid by thin layer chromotography was done by Gainer and his co-workers.[25] With this method a sample of mycophenolic acid is dissolved in CH_3OH at a concentration of 10mg/ml and 10µl of this solution is spotted on a thin layer plate. The plate is developed at room temperature, allowing the solvent front to move 15 cm from the point of application and dried at room temperature. The plate is then visualized under a UV lamp in both the short (254nm) and long (366nm) wavelenth regions. The group determined that ether-ethylether-acetic acid (80:30:5) is the best developing solvent for

separating mycophenolic acid on silica gel thin layer plates. They reported that the compound could not be separated or determined on cellulose plates with simple developing solvents.

Bopp et al. developed gas liquid chromatography and fluorometric procedures for the determination of mycophenolic acid in plasma.[26] The gas liquid chromatography procedure consists of extraction of the compound, silylation with trimethyl-silylimidazole in CS_2, and chromatography of the sample on a 2% methylphenylsilicone fluid column at 270°C. The silyl derivative of lithocholic acid is used as an internal standard. The fluorescence procedure consists of an extraction followed by measurement of the fluorescence of the compound in pH 10.0 buffer, with an excitation wavelength of 350 nm and an emission wavelength of 438 nm. This work indicates that the accuracy of both procedures is greater than 90%.

Determination of mycophenolic acid in cheese was done by Siriwardana and Lafont.[27] This method for detection of mycophenolic acid is based on selective extraction with a mixture of equal volumes of 5% sodium chloride, methanol and acetone. The pH of the solution is adjusted to 6 by addition of 1N acetic acid and precipitation of caseins is done at -25°C. Defatting is done with hexane, and removal

of extraneous matter by transfer of mycophenolic acid to chloroform, chloroform:ethyl acetate (50:50 vol/vol) and finally with ethyl acetate. The extract is purified further by column chromatography. The column material is prepared by blending 10 g of Kieselgel H with 50 ml of n-hexane:benzene (50:50 vol/vol) and further extraneous matter is eliminated by washing with benzene. Finally, mycophenolic acid is eluted with ether:n-hexane:formic acid (60:20:5, vol/vol), is visualized with diethylamine and quantitized on thin layer chromatograms by fluorescence. The limit of detection in cheese is about 20µg/kg.

Radiogas chromatography-mass spectrometry in the selected ion monitoring mode was employed as a method for the determination of mycophenolic acid by Doerfler and his co-workers.[28] The increased sensitivity and specificity of the selected ion monitoring mode detector allows straight-forward detection and identification of the relatively small formation of mycophenolic acid by Penicillium brevi-compactum.

Engel and Guenter conducted the quantitative analysis of mycophenolic acid on thin layer plates by UV-densitometric and fluorodensitometric methods.[29] In this method mycophenolic acid is obtained from cultures of Penicillium stoloniferum by chloroform extraction then crystallization and finally thin-layer chromatography.

Possibilities for UV and fluorodensitometric determination methods are evaluated. The results indicate that in ethanol solution, mycophenolic acid exhibits UV maxima at 219, 250 and 305 nm. Changes in the spectra after treatment with HCl, NaOH, and NH_3 are observed. The UV spectra of mycophenolic acid after thin layer chromatography show almost similar maxima (at 220, 250 and 300 nm) as in the ethanol solution, however, the intensities of absorption are markedly different. This work indicates that for quantitative determination, measurements at 220 nm and especially at 250 and 300 nm are suitable. UV densitometry is the method of choice, and fluorodensitometry is suggested as a confirmatory method.

Nowotny and his co-workers determined and separated mycophenolic acid in moldy food.[30] In this method, mycophenolic acid is separated and determined by thin layer chromatography (TLC) on an oxalic acid saturated silica plate with $CHCl_3$:CH_3OH (3:1) as the mobile phase. The detection limit is governed by the type of food tested and recoveries are 50 - 80%.

A method for the separation and determination of 60 antibiotics, including mycophenolic acid using paper chromatography has been reported.[31] In this method the antibiotics are first analysed in four solvent systems: distilled water, n-butanol saturated with water, ethyl

acetate saturated with water, and benzene saturated with water. The development and detection of chromatograms is done by taking strips of the Whatman Nº 1 paper 1/35 cm, the origin being 3 cm from the lower end of the strips. The standard mycophenolic acid (80 µg) is dissolved in acetone as the solvent for its solution before application on to paper strips. The result indicates that mycophenolic acid gives a distinct spot with a characteristic Rf value, which is dark green upon spraying with 3% $FeCl_3$ in methanol.

Paterson reported the identification and measurement of secondary metabolites (one of which is mycophenolic acid) in Penicillium and other fungi, by standardized one and two dimensional thin-layer chromatographic method.[32]

Frisvad and Thrane developed a general standardized method for the analysis of mycotoxins and other fungal secondary metabolites, including mycophanolic acid, based on high performance liquid chromatography (HPLC) with an alkylphenone retention index and photodiode array detection combined with TLC in two different eluents. In this method each metabolite is characterized by its alkylphenone retention time index, its UV-Vis absorption max, and its retardation factors relative to griseofulvin in 2 TLC eluents. The standards of the metabolites are dissolved in

methanol (2 ml), and no extraction or purification is done. Only the analysis of standard compounds was done by HPLC. The results of the research indicate that the retention index of mycophenolic acid is 984 and the UV maxima are 217, 249 and 302. Frisvad et al.,[33] concluded that it is very difficult, if not impossible, to propose a general effective clean up procedure for food and feeds containing trace amounts of the mycotoxins.

Noto and his co-workers determined mycophenolic acid by turbidimetric bioassay method with Candida albicans as the test organism.[34] The method's sensitivity to mycophenolic acid was affected by the composition and especially the pH of the medium, by the incubation period, and by the inoculum size. This method gave highly reproducible results. A cell suspension was added to a medium at pH 3.2 containing 0.45% yeast extract and 2% glucose in 0.1 M Na_2HPO_4 and a 0.05 M citric acid buffer. Samples (4.5 ml) were diluted with mycophenolic acid to give 0.4 - 0.2 μg of mycophenolic acid/ml. These samples were incubated at 30°C for 16 hours. One-tenth of one ml of 37% HCHO was added to each sample and the absorbance of well-shaken samples was determined at 470 nm. Responses were calculated from the absorbance and plotted versus the logarithm of mycophenolic acid concentration.

The main problem or task in the analysis of mycophenolic acid from feedstuffs and agricultural commodities is the clean up procedure. Several attempts have been made to develop reasonable clean up proceedures.

Extraction of the compound from feedstuffs and other agricultural commodities is possible with most organic solvents with minor modification. The only problem appearing in extraction is the emulsion problem.

All the investigations in the literature review deal with the measurement of the standard compound at high levels. There was no attempt to extract mycophenolic acid from samples of feedstuffs or agricultural commodities, nor was there an attempt to analyse the compound in lower concentrations such as 0.1 ppm. This investigation seeks to develop a method for determining trace amounts of the compound in feedstuffs and agricultural commodities where mycotoxins naturally occur.

EXPERIMENTAL

The following section of this thesis describes the various reagents, procedures and the instrumentation used in the research work done. Preparation of certain reagents is also discussed.

Mycophenolic Acid Standard Solutions.

Mycophenolic acid standard solutions were prepared from the standard compound purchased from Sigma Chemical Company. A 0.4 μg/μl stock standard was prepared by dissolving 2.00 mg of the standard compound in 5 ml of acetonitrile. The solution was then diluted with acetonitrile to various concentrations depending upon the type of measurement needed.

Reagents.

All solvents (methanol, chloroform, dichloromethane etc.) and the additional reagents (H_2SO_4, Na_2CO_3, $NaHCO_3$, KCl etc.) were analytical grade. Acetonitrile (CH_3CN), Fisher Scientific Co., HPLC grade was used as provided.

Samples.

All samples used in this analysis were obtained from the feed analysis lab of the Station Biochemistry

Section of the Chemistry Department, South Dakota State University. These samples include various types of hay (e.g. grass hay, alfalfa hay, oats hay, etc.) and various grains (e.g. wheat, barley, oats, corn, etc.). Samples were selected randomly to represent both hay and grain feedstuffs. The samples had been finely ground prior to receipt.

Cleaning of Glassware.

All glassware was washed in Alconox solution and then rinsed with de-ionized water, and finally left for four hours in a very hot oven (260°C).

Instrumentation Parameters.

Both high performance liquid chromatography (HPLC) and gas chromatography (GC) were found to be satisfactory methods for standard mycophenolic acid analysis. A Varian 6000 gas chromatograph with FID detector was used. A column of 3% OV-1 on 80/100 mesh with Chromosorb W-HP at 6' x 4 mm internal diameter (ID) and a column of 5% SE-52, 6' x 4 mm ID were used. For HPLC determination an ISCO V^4 UV absorbance detector with a Milton Roy mini pump, Perkin Elmer 023 recorder and a C_{18}, 25 cm x 4.6 mm ID column (Alltech Associates, Deerfield, IL) were used.

Conditions for Gas Chromatography.

Injector temperature	= 325°C
Detector temperature	= 325°C
Initial column temperature	= 150°C
Final column temperature	= 300°C
Rate of increase in temperature	= 10°C/minute
Attenuator	= 8×10^{-12}
Chart speed	= 0.5 cm/min

Conditions for High Performance Liquid Chromatography

Column - C_{18}, 25 cm x 4.6 mm ID (Alltech Associates, Deerfield, IL)

Mobil phase - 40:60 $CH_3CN:H_2O$ pH 2 with 85% H_3PO_4

Wave length - 250 nm and 300 nm

Pumping rate - 1.4 ml/min

Sensitivity - 0.01 - 0.005 absorbance units

Chart speed - 20 cm/hr

But, in the case of gas chromatography, before the standard was analyzed, methylation was necessary to make the compound volatile. The methylation was done with diazomethane which was prepared from N-methyl-N-nitros-P-toluene sulfonamide. The methylation was done by mixing 1 ml of the 0.4 µg/µl standard and 1 ml of diazomethane,

letting them stand for about 15 minutes and then evaporating under N_2 gas until the last drop. Finally the last drop was dissolved in 2 ml HPLC grade acetonitrile for GC analysis.

Gas chromatography worked well with the standard mycophenolic acid, but with the extracts of hay and grain samples excessive background interfered with the analysis of the compound sought. However, the high performance liquid chromatography system worked reasonably well with both the standard and the extracts of hay and grain. So, HPLC was selected as the analytical method of choice.

Treatment and Analysis of Standards.

First 5 µl of the 0.4 µg/µl standard was injected in the HPLC with a 10 µl syringe and the peak was found to go off the scale of the chart paper. Then appropriate dilutions were made to 0.04 µg/µl and to 0.004 µg/µl whenever it was necessary using acetonitrile.

The mobile phase in the high performance liquid chromatography system was 40:60 acetonitrile:H_2O (pH 2). Both the acetonitrile and the glass distilled water were filtered with suction to remove dust and other particles which can block the column. This composition (40:60 CH_3CN:H_2O) of the mobile phase was determined by trial and error. First 70:30, 60:40, and 50:50 acetonitrile:water

(pH 2) were tried. At these compositions the compound eluted so quickly that separation and resolution of peaks were not good enough. Then the 40:60 acetonitrile:water (pH 2) was tried. With this ratio the mycophenolic acid peak came out very slowly, so it was decided to use the 40:60 acetonitrile:water composition ratio as the mobile phase.

Extraction.

Feed or ground grain samples of 30 gm were extracted with 150 ml of 90:10 methanol:water and 30 ml of saturated potassium chloride solution by mechanical shaker for 10 - 30 minutes. The extract was filtered through fluted filter paper and a measured amount of filtrate taken (eg. 125 ml for hay samples and 125 ml for grain samples). After the extraction, it was necessary to clean the extract, and for this purpose two procedures were developed.

a) Column Chromatography.

The extract was first partitioned twice with 75 ml portions of hexane, discarding the hexane layers. Water (75 ml) was added to the 125 ml filtrate (extract), and the aqueous methanol extract was partitioned with 3 x 50 ml portions of dichloromethane. The lower dichloro-methane layers were combined and taken to dryness

on a rotary evaporator. The residue was brought up to 3 ml in a test tube with dichloromethane for the column. The column was prepared by 15 g silica gel, SilicAr CC-4 of 200 - 325 mesh, (Mallinckrodt, Paris, KT) and 50 ml chloroform. The 3 ml extract was added to the column and 100 ml of chloroform was added to elute some of the impurities. Finally mycophenolic acid was eluteted with 150 ml of dichloromethane:acetic acid (98:3, vol/vol). The mycophenolic acid fraction was evaporated on a rotary evaporator first, then concentrated to the last drop under nitrogen, finally the last drop was dissolved with 2 ml of HPLC grade acetonitrile for analysis.

In this clean up procedure, separation on alumina and Florisil were attempted, but both were found to strongly retain the compound in the column and it was decided to use silica gel as the only adsorbent. The possibility of using other brands of silica gel (other than SilicAr CC-4) and the effect of mesh size change were studied. A silica gel brand purchased from Sigma Chemical Company (St. Louis, MO) with mesh size 28 - 200, mean pore diameter:22Å, and two other brands purchased from Millinckrodt (silicic acid) of mesh size 100 and 325 were tried. The latter brands did not work at all, but the former one (the one from Sigma Company) worked upon deactivation by 5% H_2O.

b) Ligand Exchange

On the basis of the work done by Petronio and his co-workers[35] which was ligand exchange on column chromatography, an attempt was made to apply the principle as a clean up method for the extract. In this method IR-120 resin Amberlite (30 - 50 mesh) was used as ion exchange resin in the Fe^{+3} form. Both the resin and $Fe_2(SO_4)_3$ were mixed in a beaker, then introduced in column followed by 1 ml of the 0.4 µg/µl standard. The column was washed with different solvents and other different reagent solutions as needed, but this adsorption method did not work. The effect of different solvents and reagent solutions on this column was studied.

c) Acid-Base Partitioning

The filtrate was made basic (pH 9) by 10% Na_2CO_3 (immediately after extraction), and neutral interferences were removed by 2 extractions with 50 ml chloroform, discarding the chloroform layer. Then the filtrate was made acidic (pH 2) by H_2SO_4 and since the mycophenolic acid was neutral at this pH, it was extracted three times with 50 ml chloroform and the aqueous methanol layer was discarded. Finally, the extract was evaporated on a rotary evaporator, concentrated to the last drop under nitrogen gas, then the last drop was dissolved with 2 ml of HPLC

grade acetonitrile for analysis.

In addition to these extraction and clean up methods two other procedures: a) extraction into slightly alkaline H_2O, then acidification and finally extraction into organic solvent, and b) extraction into 100% pure water, then partitioning into CH_2Cl_2, $CHCl_3$ etc. were tried and studied as far as their shortcomings and advantages.

Data Analysis

In this investigation, the amount of mycophenolic acid recovered (% recovery), after all the extraction, partitioning and clean up steps was calculated using the following equations:

$$\% \text{ recovery} = \frac{\text{ppm found}}{\text{ppm added}} \times 100 \qquad (1)$$

$$\text{ppm added} = \frac{(\text{V of std inj in } \mu\text{l}) \times (\text{Conc of std in } \mu\text{g}/\mu\text{l})}{(\text{wt of sample in g})} \qquad (2)$$

$$\text{ppm found} = \frac{(\text{V of sample extract in ml}) \times (\text{ng of std inj}) \times (\text{peak ht of sample})}{(\text{wt of sample in g}) \times (\text{V of sample inj}) \times (\text{peak ht of std})} \qquad (3)$$

V = volume in µl or ml wt = weight in grams ht = height

ng = nanogram Std = standard inj = injected

RESULTS AND DISCUSSION

This part of the thesis describes results obtained from proceedures described in the experimental section. The optimum instrumental parameters and conditions for analysis are discussed. Conditions of maximum efficiency for extraction are also discussed. In addition, the shortcomings of certain methods and procedures are presented.

Instrumental Parameters.

As was indicated in the experimental section of this thesis, both high performance liquid chromatography (HPLC) and gas chromatography (GC) were used in the analysis of mycophenolic acid standards under the conditions specified in the experimental section. Both methods provided a reasonable resolution of peaks with a reasonable retention time as far as the analysis of the standard compound is concerned. Typical chromatograms for both HPLC and GC are illustrated in Fig. 3 and Fig. 4 respectively.

Gas chromatography worked well with the mycophenolic acid standard, but when the spiked extracts of hay and grain samples were tried, excessive background interfered with the analysis of the compound sought. A

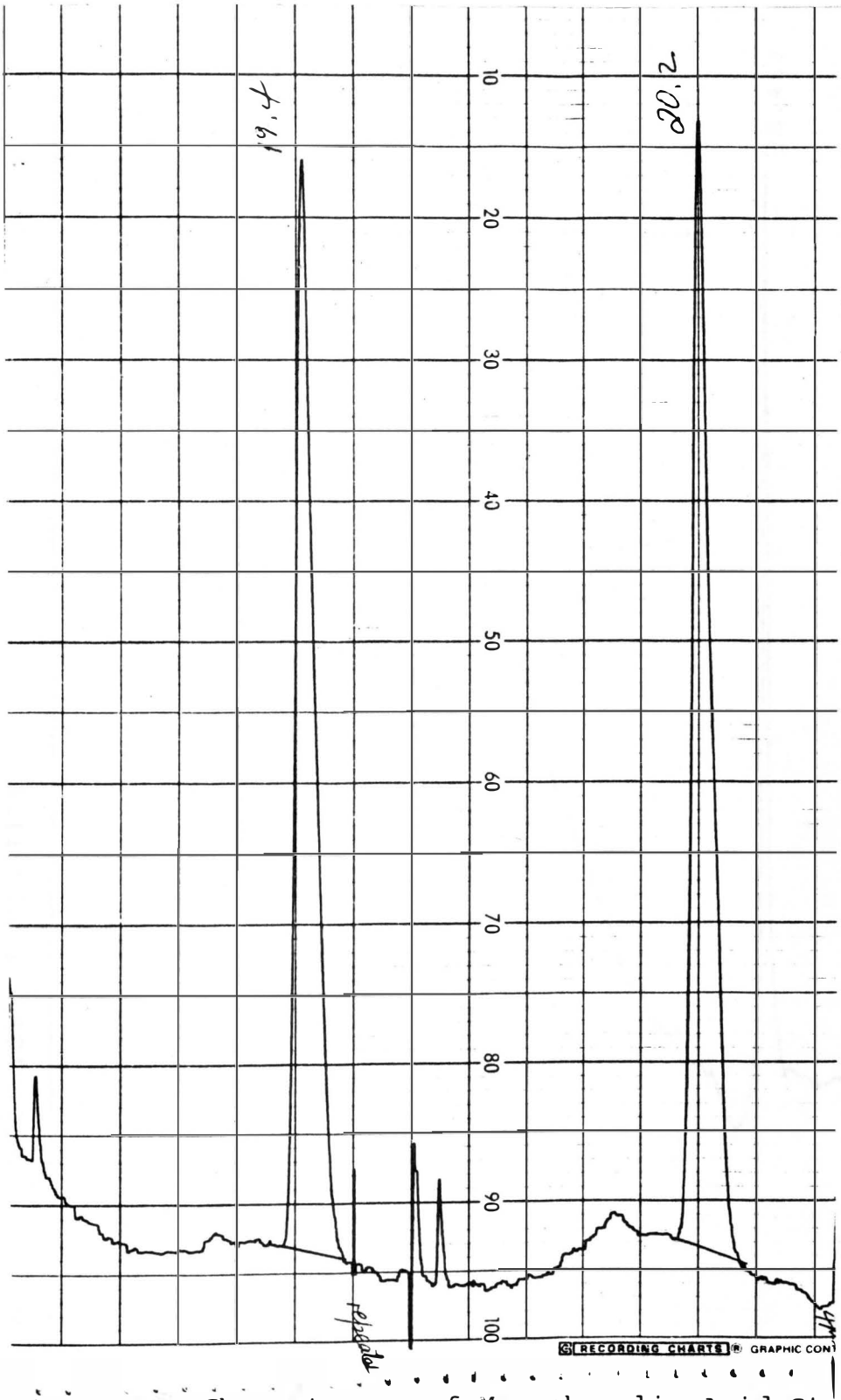

Figure 3. Chromatogram of Mycophenolic Acid Standard by HPLC

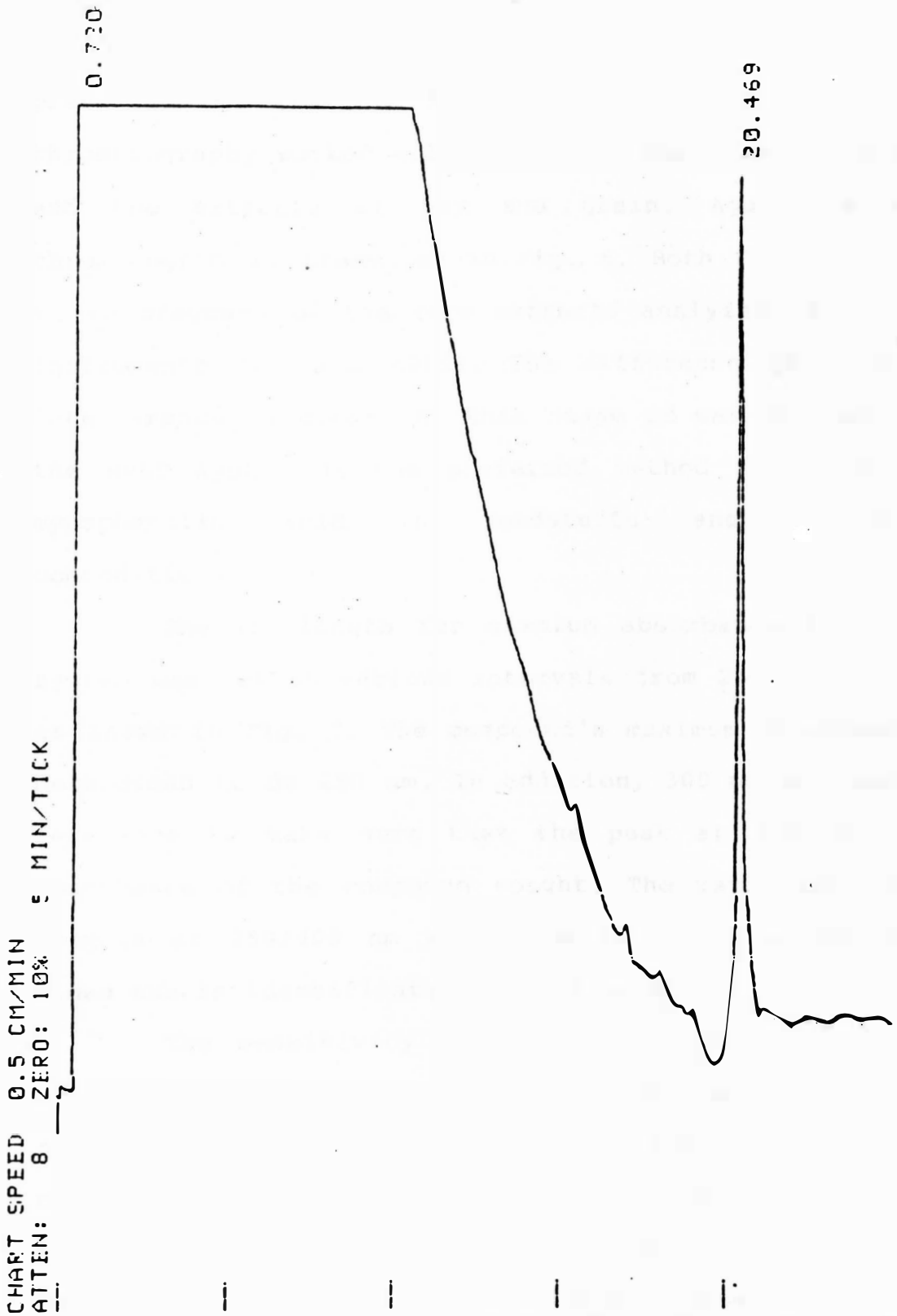

Figure 4. Chromatogram of Mycophenolic Acid Standard by GC

sample chromatogram which illustrates this fact is presented in Fig. 5. However, high performance liquid chromatography worked well with both the standard compound and the extracts of hay and grain. Again, a sample chromatogram is presented in Fig. 6. Both Fig. 5 and Fig. 6 are diagrams of the same extracts analyzed by different instruments (GC and HPLC). The difference in background interference is clear. At this stage it was decided to use the HPLC system as the preferred method for determining mycophenolic acid in feedstuffs and agricultural commodities.

The wavelength for maxmium absorbance in the HPLC system was set at various intervals from 240 nm to 305 nm as shown in Fig. 7. The compound's maximum absorbance was determined to be 250 nm. In addition, 300 nm was used as a reference to make sure that the peak at 250 nm is the absorbance of the compound sought. The ratio of the peak heights at 250/300 nm was found to be 2, which greatly aided the in identification of the peak.

The sensitivity setting of the detector used for most of the analyses of the compound was 0.005. In the sensitivity adjustment, the greater the sensitivity, the more noisy the base line, and the less the sensitivity the less intense are the peaks. This low intensity makes analysis of the peaks difficult. The height of the

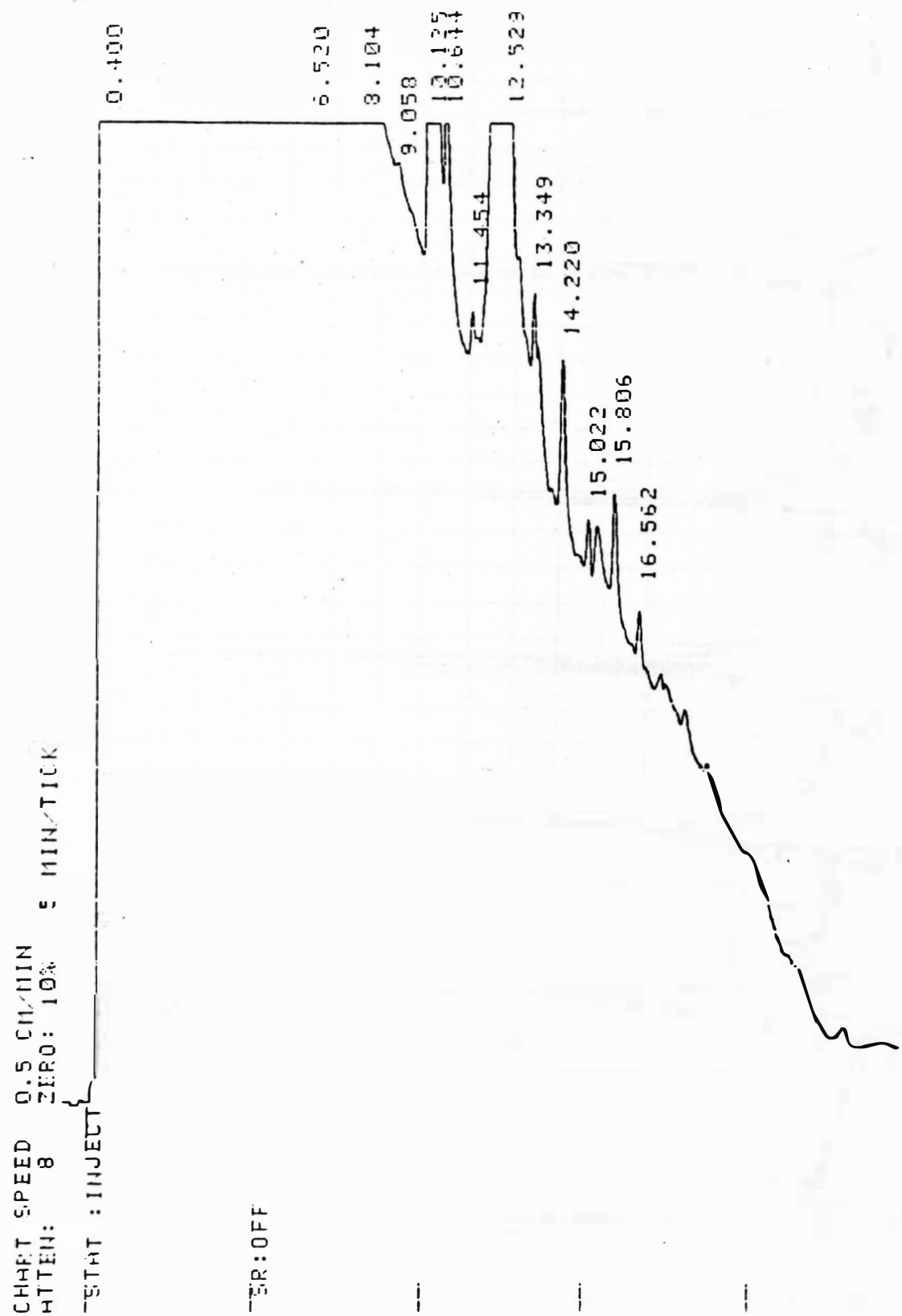

Figure 5. Chromatogram of Feedstuffs' Extract by GC

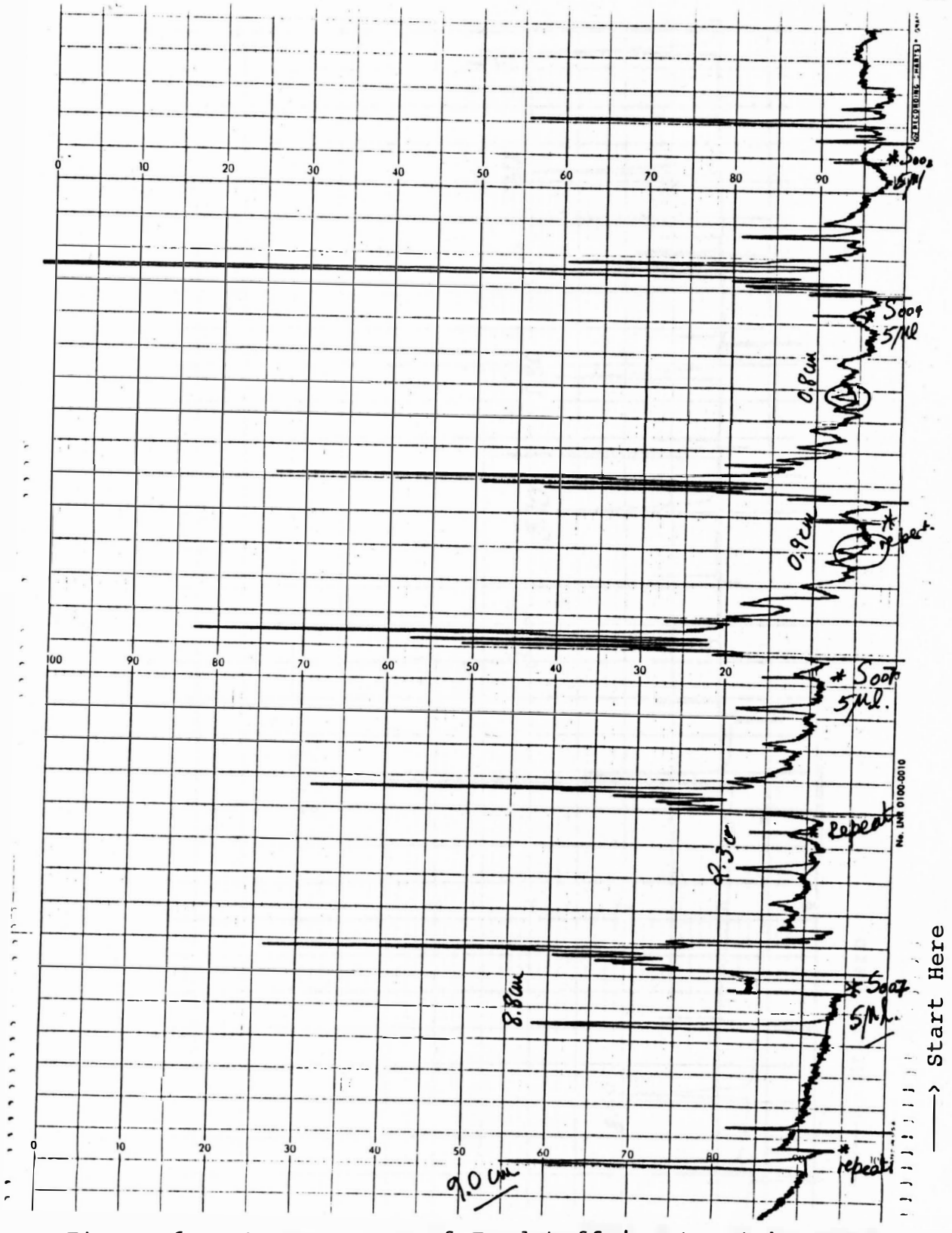

Figure 6. Chromatogram of Feedstuffs' Extract by HPLC

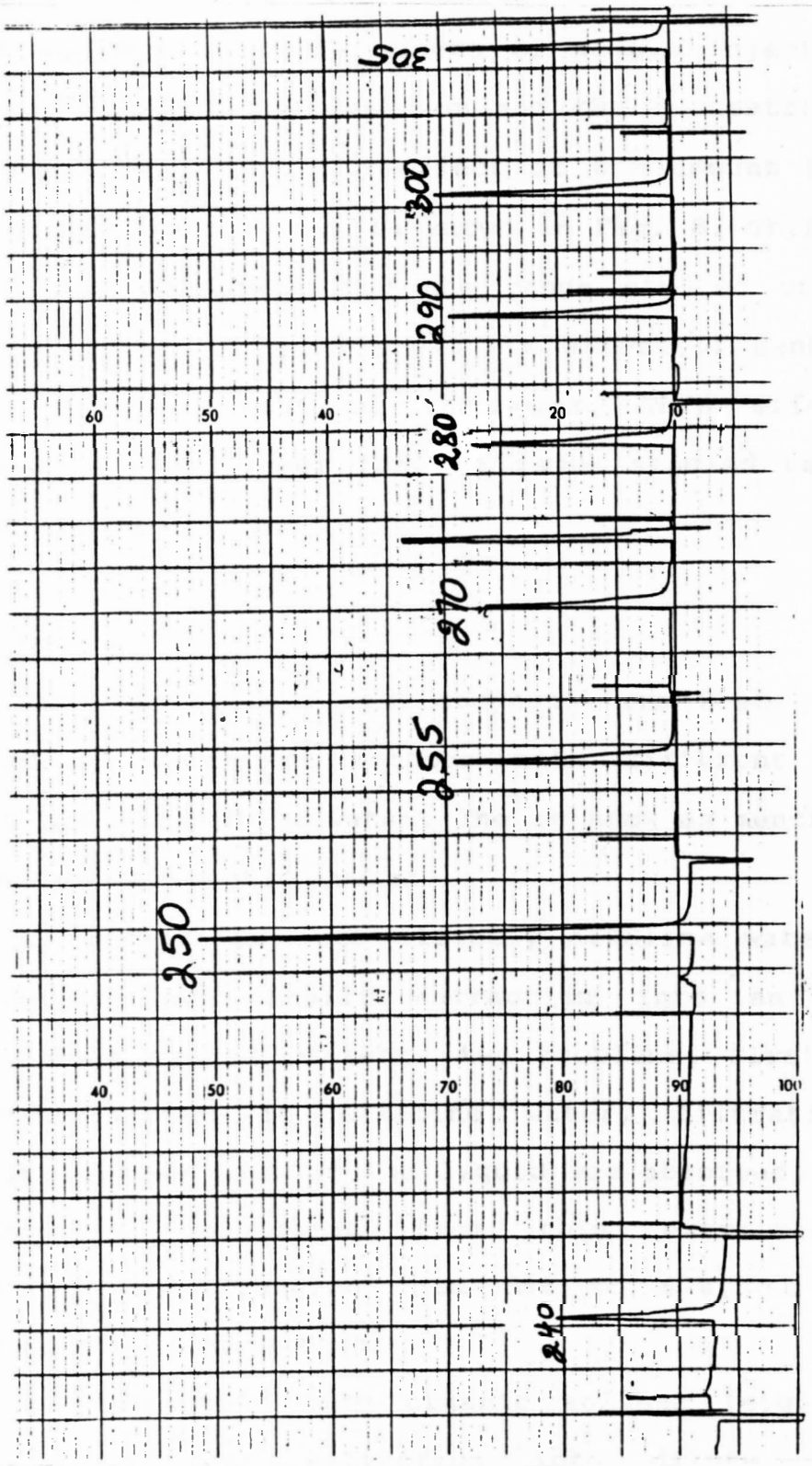

Figure 7. Scanning of Absorption Maxima of Mycophenolic Acid

peak is dependent on the amount of sample injected, the sensitivity set of the detector and the concentration of the standard solution. The effect of the amount injected on the height of the peak is shown in Fig. 8. This effect was found to be linear for the range studied under the same sensitivity. Fig. 9 shows the effect of sensitivity setting on the height of the peak. This effect was also found to be linear for the range studied under the constant amount injected.

Extraction

The extraction of the compound from feedstuffs was attempted by different methods with different organic solvents and with plain water. The methods as mentioned in the experimental section were:

a) Extraction into slightly alkaline water, then acidification and finally extraction into an organic solvent. This method presents two problems. First, since it was extracted in alkaline water, filtration was difficult because most of the liquid was absorbed. Second, the recovery calculated from this extraction was unacceptably low, ranging from 58% to 67%, so it was decided to drop this method.

b) Extraction into organic solvent (e.g. $CHCl_3$, CH_2Cl_2, etc.) then extraction into dilute alkaline

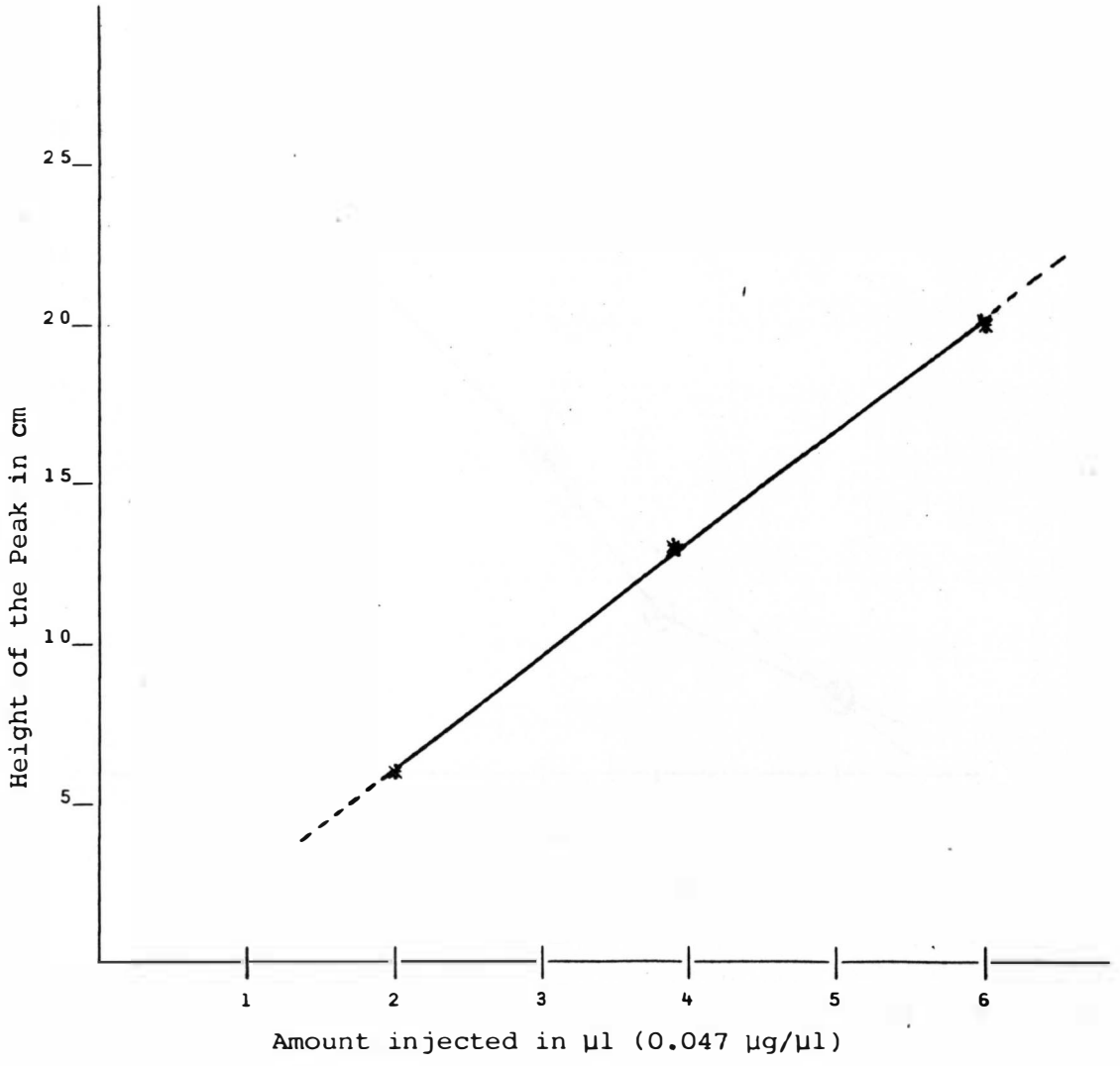

Figure 8. Effect of Amount Injected on the Height of the Peak

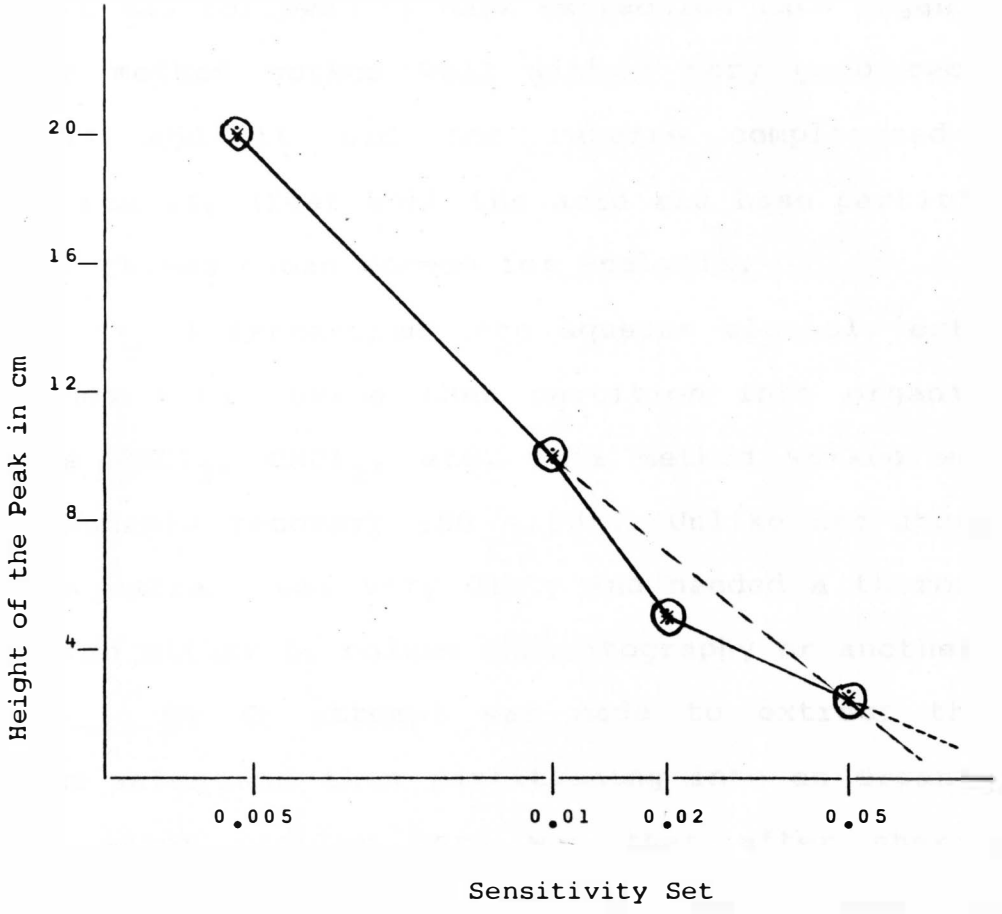

Figure 9. The Effect of Sensitivity Setting on the Height of the Peak

solution, and finally the acidification of the aqueous phase was followed by back extraction into organic solvent. This method worked well with a very good recovery (85% -95%) and it did not require complicated clean up procedures. After both the acid and base partitioning, the extract was clean enough for analysis.

c) Extraction into aqueous alcohol, extraction of lipids with hexane then partition into organic solvents like $CHCl_3$, $CHCl_2$, etc. This method worked well with a reasonable recovery (80 - 90%). Unlike the above methods, this extract was very dirty and needed a thorough purification either by column chromatography or another method.

d) An attempt was made to extract the compound from water and then partitioning into an organic solvent. The major problem here was that after shaking in the mechanical shaker, filtration was almost impossible. Filtration was attempted with filter aid (Celite) but was unsuccessful. Thus, this method was not developed further.

At this point it can be seen that methods (b) and (c) were taken as the accepted methods of extraction. Method (b) has a slightly higher recovery and an easier procedure, but method (c) has a reasonable recovery with much cleaner extracts.

In extracting the compound from feedstuffs and other agricultural commodities, different organic solvents

were tried, but due to their shortcomings in the extraction efficiencies, only a few were used after trial and error tests. Some of the solvents were found to be inapplicable due to emulsion problems (eg. ethyl acetate) and for some of them (e.g. acetonitrile) the recoveries were not high enough to be accepted as good extraction solvents. Of the solvents used in the extraction of the compound, the ones with a good recovery and fewer emulsion problems were methanol acetonitrile for method two.

In both extraction methods (b) and (c), it was found that addition of KCl solution reduced the solubility of organic solvents in water, and prevented the emulsion formation. An attempt was made to extract the compound using organic solvents in the absence of KCl solution, but it was nearly impossible to deal with the emulsion. Also, it was determined that the addition of water in a volume equal to that of the extract immediately after filtration was important in preventing emulsions. This was particularly critical for grain extracts.

The time taken for filtration and the amount of extract collected are related to the amount of KCl solution added. This is because the water added along with the KCl solution increases the filtration time (for grain samples) and decreases the volume of extract collected (for hay samples).

Cleaning the extract

As was indicated in the experimental section the extract was so dirty that separation and identification of the sought compound or analyte was almost impossible. A sample chromatogram is presented in Fig. 10. At this stage it was realized that a clean up procedure was necessary, and on this basis two clean up procedures were developed.

a) Column Chromatography

The first method tried was based on column chromatography. The extract to be cleaned by column was first partitioned with hexane to remove the fatty materials from the extract. This defatting step was most important for the grain samples. Then, in order to remove some of the interference in the aqueous phase, the extract was partitioned three times with chloroform. After concentrating the chloroform extract layers to 3 ml, it was introduced onto the column. The solvents used in the column chromatography were chosen after trial and error on thin layer chromatography. The solvents tried were:

a) ethyl acetate, ethyl acetate:acetic acid 9:1

b) toluene, toluene:acetic acid 9:1

c) chloroform, dichloromethane:acetic acid 98:2

Of these, only the chloroform, dichloromethane:acetic acid series was found to be useful. The chloroform was used for

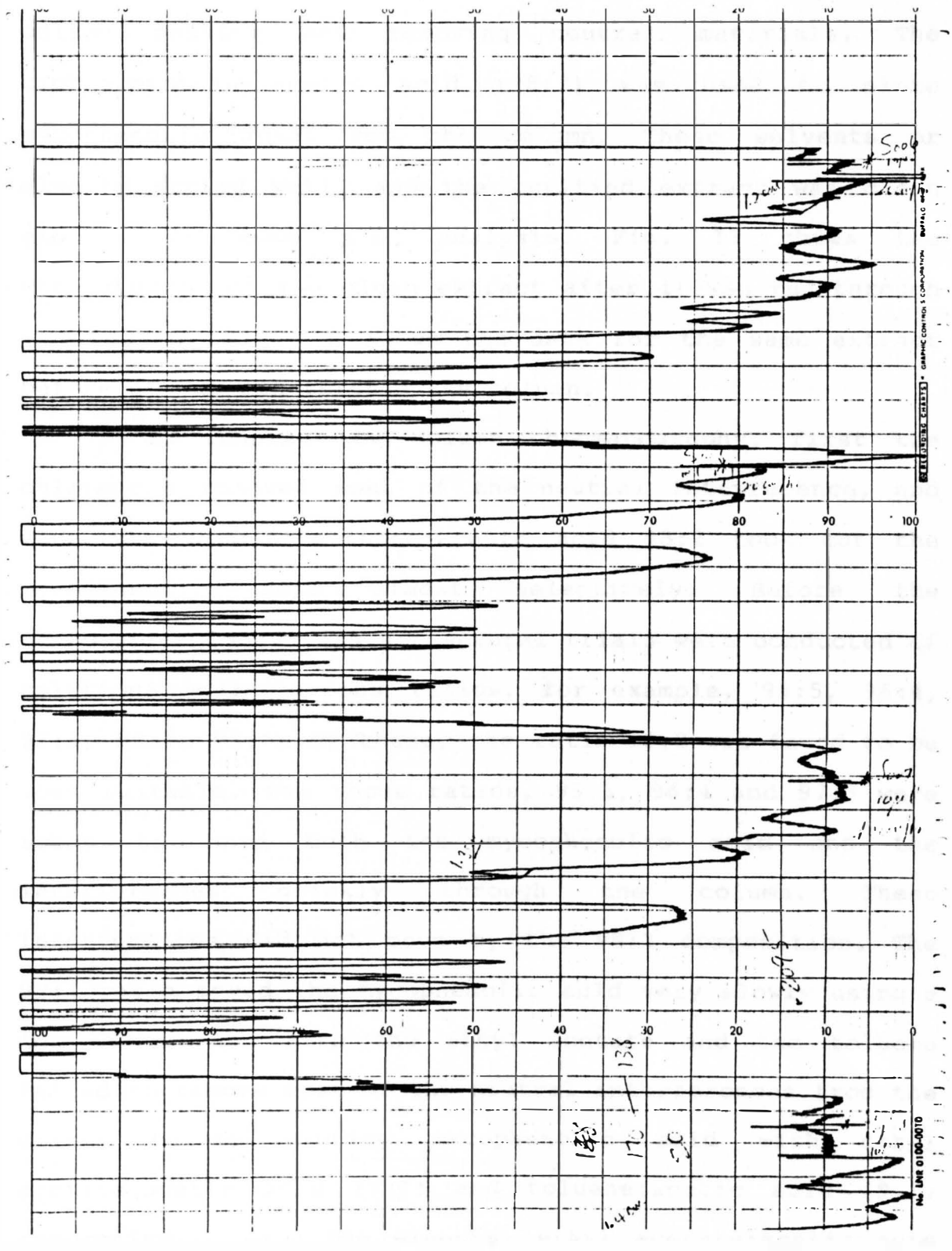

Figure 10. Sample Chromatogram of Uncleaned Extract

column packing and removing neutral materials. The dichloromethane:acetic acid (98:2) was used to elute mycophenolic acid from the column. These solvents or eluents worked well, and the purified extract was clean enough for good HPLC analysis. Fig. 11 shows the chromatogram of the clean extract after it was run through the column. Fig. 10 shows the data for the same extract before it was run through the column.

In running the column chromatography, first the chloroform removed most of the neutral interference, and then the dichloromethane:acetic acid 98:2 took out the mycophenolic acid almost selectively. Before the composition (98:2) was set several trials were conducted of solutions with various ratios, for example, 95:5, 96:4, 97:3, 98:1, 99:1. Of these, the ratio 98:2 was found to be most suitable. The three ratios, 95:5, 96:4 and 97:3 were found to move both the mycophenolic acid and the interferences quickly through the column. These interferences did not move by the 98:2 composition. The 99:1 ratio moved the mycophenolic acid very slowly using a lot of eluent. Both the ethyl acetate and the toluene failed to remove most of the neutral interferences from the column before eluting mycophenolic acid with ethyl acetate:acetic acid (9:1) and toluene:acetic acid (9:1) respectively. Both the eluents, ethyl acetate:acetic acid

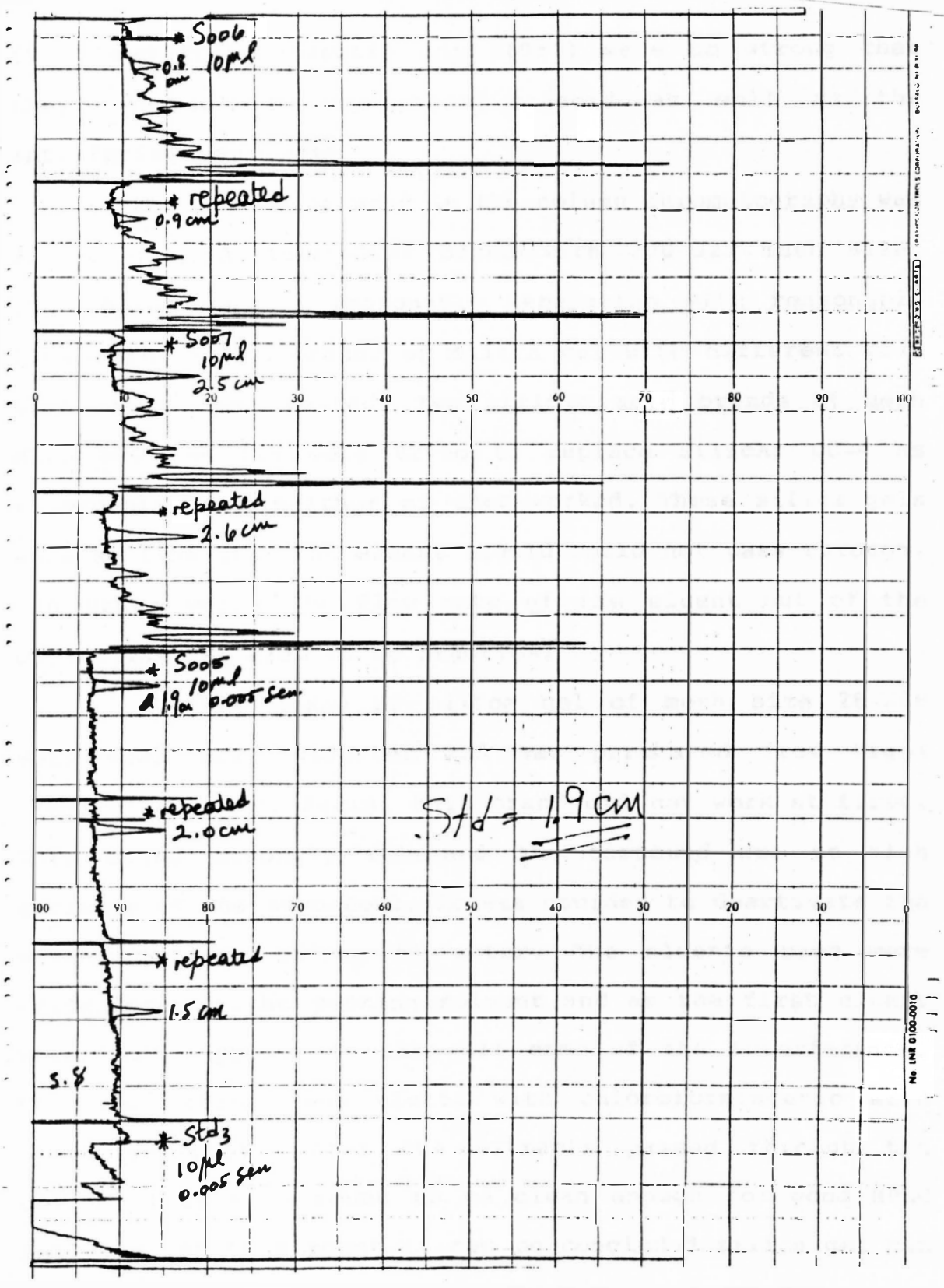

Figure 11. Sample Chromatogram of Cleaned Extract

(9:1) and toluene:acetic acid (9:1) were so strong that they removed the mycophenolic acid as well as the interfering compounds.

The adsorbent used in the column chromatography was silica gel, SilicAr CC-4 brand with 200-325 mesh size. This brand gave a reasonable separation with reasonable flow rate. Other brands of silica gel with different mesh sizes were also tested. Two silicic acid brands of mesh size 100 and 125 were tried to replace SilicAr CC-4 as adsorbents, but neither of them worked. These silica gels were so fine that the eluent liquid could not pass through. In other words the flow rate of the eluent out of the column was too slow to be practical.

Another brand of silica gel of mesh size 28-200 with mean pore diameter 22Å was purchased from Sigma Chemical Company. Again, this brand did not work at first. This brand strongly retained the compound due to high activity of the adsorbent. It was decided to deactivate the silica gel by adding 5% water. The eluents used were chloroform as the packing solvent and as the first eluent which was supposed to take off some of the interference. Then the column was eluted with chloroform:acetic acid (95:5 vol/vol). After the extracts passed through the column, they were found to be clean enough for good HPLC analysis. At this point it can be concluded silica gel can

be used as an adsorbent in the partitioning of the extracts of both hay and grain samples with certain modifications, such as deactivation. Results of the grain and hay extracts with their recoveries after running through the column are presented on Tables 1 and 2.

As indicated in the experimental section, separation on alumina and Florisil were attempted, but neither of them was found to work because both adsorbents retained the compound strongly. In both cases eluents of different strength were used as follows:

a) Chloroform

b) dichloromethane:methanol 90:10 v/v

c) dichloromethane:methanol 75:25 v/v

d) dichloromethane:methanol:acetic acid 73:25:2 v/v

All the eluents were collected, evaporated and concentrated to the appropriate volume, but none of them gave the peak of mycophenolic acid upon HPLC analysis. In fact, the evaporated and concentrated eluents of alumina from (c) and (d) showed a very broad peak. For Florisil, only eluent number (d) gave the broad peak. At this point, it was decided not to use them as adsorbents.

b) Ligand Exchange.

Besides the adsorption column chromatography, adsorption by ligand exchange was tried. The idea behind

Table 1: Recoveries and Detection Limits of Grain Extracts

Type of feed sample	recovery	standard added
wheat	94.6%	5 ppm
winter wheat	83.4%	2 ppm
sorghum	83.2%	1 ppm
barley	92.0%	2 ppm
corn	87.2%	0.5 ppm
wheat	86.0%	0.1 ppm
wheat	88.5%	1 ppm
barley	90.5%	5 ppm
corn	89.5%	0.5 ppm

The mean or average recovery = 88.32%
Minimum detection limit = 0.1 ppm
Standard deviation = 3.81

Table 2: Recoveries and Detection Limits of Hay Extracts

Type of feed sample	recovery	standard added
alfalfa hay	78.8%	5 ppm
grass hay	70.6%	6 ppm
pig hay	72.5%	5 ppm
grass/alfalfa hay	73.7%	1 ppm
grass/alfalfa hay	73.4%	0.5 ppm
grass hay	70.0%	2 ppm
alfalfa hay	75.6%	1 ppm
alfalfa hay	74.0%	2 ppm
grass hay	69.0%	1 ppm

The mean or average recovery = 72.26%
Minimum detection limit = 0.5 ppm
Standard deviation = 3.58

this was, by taking ion exchange resin in the form of Fe^{+3}, and assuming mycophenolic acid as a strong phenolic compound to keep mycophenolic acid in the column attached to Fe^{+3} through its phenolic group and to flush all interferences of the extract with strong solvent. Then after washing the column, to exchange the phenolic group of mycophenolic acid by another ligand of stronger affinity to Fe+ and finally to remove the interface free mycophenic acid from the column using appropriate eluent. Since the phenolic group of mycophenolic acid is weak, it failed to remain attached to the Fe^{+3}. It came out of the column right away after a fairly nonpolar solvent. At this point it was decided not to pursue this method further.

c) Acid-Base Partitioning.

Clean up procedure number two was based on acid-base partitioning. The filtrate was made basic (pH 9) by sodium carbonate solution immediately after extraction and then partitioned twice with chloroform to remove neutral interferences and by acidifing the extract, mycophenolic acid returned to its neutral state and was extracted three times with chloroform and the non-neutral interferences were discarded with the aqueous phase. Upon analysis of the extract on HPLC, it gave a reasonable resolution of the peaks with quite less interference

background especially with the grain samples analysis was possible without further cleaning by column chromatography. Of course, the cleaning by acid base partitioning is not really satisfactory, especially with the hay samples, it is almost impossible to analyze without clean up, but much better than the totally uncleaned ones.

Even though the recovery and the cleanliness of the extract is highly dependent upon the type of samples used (for example hay and grain samples or even two hay samples do not have the same recovery). Anyway out of the two different clean up procedures discussed in this section, the acid-base partitioning was found to have a little bit higher recovery. This difference in recovery may be explained by the loss of the compound in the column. On the other hand, the extract came out through the column was with much less background interference than the one cleaned up by acid-base partitioning especially for hay samples. This result may be explained by the fact that a lot of interferences have the same acid-base character as mycophenolic acid does. To generalize, the acid-base partitioning clean up is not satisfactory, so column chromatography almost always necessary for cleaning the extracts. The slight decrease in recovery is preferred to the messy background.

CONCLUSION

An analytical method for the trace determination of mycophenolic acid has been investigated. The optimum instrumental parameters were presented with their recovery.

The recoveries for the extract cleaned up by acid-base partitioning and by column chromatography was found to be in the range of 85 to 95% for the grain samples and 70 to 80% for the hay samples. Even though the cleaning by acid-base partitioning method is a lot easier than the column chromatography cleaning method, still the latter was absolutely necessary especially for hay extracts. The extracts cleaned up by acid-base partitioning can not be injected beyond certain volume limits (due to messy background interference). As the result the detection limit is lower than the one cleaned by column chromatography.

The detection limit for grain and hay cleaned by column chromatography was 0.1 ppm and 0.5 ppm respectively. On the other hand for the extracts cleaned up by acid-base partitioning was 0.5 ppm and 1 ppm for grain and hay samples respectively. Detection limit is defined as the concentration necessary to give a signal-to-noise ratio of 2.

The two brands of silica gel used gave different backgrounds i.e. the column used the silica cc-4 brand

gave much more clean extracts than the other one, but with a little bit lower recovery (not more than 2-4%).

Recoveries and purity of extracts was highly dependent upon the type of sample extracted and the type of cleaning procedure used.

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